

# Characterization of Polyphenol Oxidase from Walnut

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**ABSTRACT.** The enzyme polyphenol oxidase (PPO) is nearly ubiquitous in Kingdom Plantae and catalyzes the oxidation of phenolic compounds into highly reactive quinones. Although the functional importance of PPO in plants remains uncertain, a putative antipathogen role for walnut (*Juglans regia*) PPO was posited as early as 1911. However, despite the rich diversity of phenolics present in walnut leaves and hulls, walnut PPO has been little studied since the early 1900s. We cloned a PPO-encoding gene from a walnut pistillate flower cDNA library and designated the gene *jrPPO1*. Genomic Southern analysis demonstrated that *jrPPO1* is the sole PPO gene in walnut. Transgenic tobacco (*Nicotiana tabacum*) plants expressing *jrPPO1* display greater than 10-fold increases in leaf PPO activity compared with wild-type tobacco, demonstrating that *jrPPO1* encodes a functional enzyme. The *jrPPO1* protein is expressed primarily in the leaves, hulls, and flowers of walnut trees and is not regulated by wounding or methyl jasmonate. To examine whether walnut PPO could affect pathogen resistance, tobacco plants expressing *jrPPO1* were challenged with *Pseudomonas syringae* pv. *tabaci*. Based on both symptom development and quantitative analyses of bacterial growth in planta, the PPO-expressing plants did not display increased resistance to this pathogen. Leaf extract browning assays indicated that tobacco leaves lack the endogenous phenolic substrates required for significant *jrPPO1* activity and quinone production in planta.

Polyphenol oxidase (PPO) is a Type 3 copper protein, which catalyzes the oxidation of monophenols or *o*-diphenols to *o*-quinones (Klabunde et al., 1998). PPO-generated quinones are highly reactive and will crosslink with proteins or polymerize, generating dark-colored melanins (Walker and Ferrar, 1998). PPO is of substantial commercial interest as a result of its central role in postharvest browning of a variety of crops, including apple (*Malus domestica*), potato (*Solanum tuberosum*), and pineapple (*Ananas comosus*) (Steffens et al., 1994). In intact plant cells, plastid-localized PPO is physically separated from its phenolic substrates, which reside primarily in the vacuole. Thus, PPO activity is generally observed only on loss of cellular compartmentalization caused by senescence, wounding, or other tissue damage (Steffens et al., 1994).

Characterized PPO isoforms in plants range from ≈51 to 73 kDa with preproteins often undergoing substantial posttranslational modifications in the form of N-terminal plastid transit peptide processing and C-terminal proteolysis (Thipyapong et al., 2007; VanGelder et al., 1997). All PPOs possess two highly conserved copper-binding domains (CuA, CuB), each containing several canonical histidine residues that form a Cu<sup>2+</sup> binding site (Klabunde et al., 1998). PPO is often isolated from plant tissues in a latent form, which can be activated by a range of treatments, including proteolysis, aging, and incubation with detergents. Unlike animal and fungal PPOs (tyrosinases), many plant PPOs lack monophenol oxidase (cresolase) activity, limiting potential substrates to diphenolic compounds such as

catechol, 3,4-dihydroxyphenylalanine, and chlorogenic acid (Steffens et al., 1994).

PPOs are often, but not exclusively, encoded by multigene families in plants. For example, seven PPO-encoding genes have been identified in tomato (*Solanum lycopersicum*) and six PPO genes are in the potato genome, whereas PPO appears to be encoded by a single gene in grape (*Vitis vinifera*) and lettuce (*Lactuca sativa*) (Thipyapong et al., 2007). Characterized PPO-encoding genes in plants are generally ≈2 kb in length and lack introns. They often display distinct tissue-specific and developmental regulation with young leaves, fruit, and flowers possessing the highest PPO transcript levels in several different species (Steffens et al., 1994). In some plants, most notably tobacco, tomato, and hybrid poplar (*Populus trichocarpa* × *P. deltoides*), PPO transcription and activity is upregulated in leaves by wounding and the wounding-associated hormones methyl jasmonate and systemin (Constabel and Ryan, 1998; Constabel et al., 2000; Thipyapong and Steffens, 1997).

PPO has several well-defined roles in animals, including skin/exoskeleton pigmentation and cuticle sclerotization (Steffens et al., 1994). In plants, however, the general physiological functions of PPO are substantially less clear, because most PPO research has focused on the agricultural implications of PPO-mediated postharvest browning. Several specialized PPO enzymes have been shown to be involved in the biosynthesis of aurone pigments in snapdragon (*Antirrhinum majus*) (Nakayama et al., 2001), betalain pigments in pokeweed (*Phytolacca americana*) (Gandia-Herrero et al., 2005; Joy et al., 1995), and 8–8' linked lignans in creosote bush (*Larrea tridentata*) (Cho et al., 2003). In addition, a protective function of PPOs has long been hypothesized (Cook et al., 1911; Kosuge, 1969), and several recent studies have provided strong support for a role in plant defense against pathogens and

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insects. In some plant species, insect herbivory strongly induces PPO activity in vivo (Constabel et al., 2000; Ruuhola et al., 2008), and the larvae of forest tent caterpillar (*Malacosoma disstria*) and common cutworm (*Spodoptera litura*) display increased mortality and decreased growth when fed leaves of plants overexpressing PPO (Mahani et al., 2008; Wang and Constabel, 2004). Similarly, viral, bacterial, and fungal challenge can induce PPO activity (Mayer and Harel, 1979), and PPO-overexpressing tomatoes display enhanced resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Li and Steffens, 2002). Protective effects of PPO have generally been attributed to the generation of reactive quinones, which may 1) possess direct bacteriocidal/insecticidal properties (Vaughn et al., 1988); 2) generate toxic reactive oxygen species through secondary oxidation reactions (Steffens et al., 1994); 3) reduce protein palatability and digestibility by oxidizing nucleophilic amino acids (Felton et al., 1992); and 4) form an impermeable melanin barrier, preventing the spread of pathogen infection (VanGelder et al., 1997).

Walnut possesses an exceptionally abundant and diverse array of polyphenolic compounds (Colaric et al., 2005; Gupta et al., 1972), some of which may play a role in pathogen resistance (Radix et al., 1998; Solar et al., 2006). Thus, walnut presents an especially interesting system for the study of PPO regulation and function. Very early work suggested that “poly-atomic phenol oxidase” activity in walnut hulls produced a germicidal fluid that could inhibit bacterial growth (Cook et al., 1911). More recent studies have described the thermostability and basic biochemical properties of PPO activity in walnut hull extracts (Mchedlishvili et al., 2005; Piffaut and Metche, 1991). Overall, however, very little is known about the biochemistry and molecular biology of PPOs in walnut. In this article, we describe the cloning of the single gene encoding PPO in walnut, the regulation of walnut PPO in planta, and the characterization of transgenic plant lines overexpressing walnut PPO.

## Materials and Methods

**PLANT MATERIAL AND TREATMENTS.** Walnut (cv. Chandler) tissues were harvested from field-grown trees at the University of California, Davis and were transported to the laboratory on ice. Kernel, pellicle, and hull tissues were removed from  $\approx$ 5-cm long mature fruit before hull split. For methyl jasmonate treatments, hulls and leaves were sprayed to runoff with a 0.025% methyl jasmonate solution in water/0.1% Triton X-100 (Dow Chemical, Midland, MI) (Constabel and Ryan, 1998). The methyl jasmonate treatment was repeated after 24 h, and tissue was collected 48 h after the first treatment. Wounding treatments consisted of crushing the leaf midvein with forceps and repeated piercing of the leaf lamina or fruit hull with a 21-G needle. Wounded tissue was collected 48 h after treatment. Homozygous T2 generation tobacco plants (cv. Petit Havana SR1) transformed with *jrPPO1* were grown from seed in a glasshouse under ambient light conditions with air temperature maintained below 28 °C with an evaporative cooler.

**POLYPHENOL OXIDASE ASSAYS.** Total protein was isolated from walnut and tobacco tissues by homogenization in extraction buffer D [50 mM Tris base (pH 8.3), 11% glycerol, 1% PEG 8000, 0.015% citric acid monohydrate, 0.010% cysteine monohydrate, and 0.010% ascorbic acid]. Homogenization was performed using a 12:1 ratio of buffer (mL) to tissue (g) and 0.5 g of insoluble PVP per gram tissue was included. After

complete homogenization, extracts were centrifuged (20,000  $g_n$ , 4 °C, 20 min), and the supernatants were stored at –80 °C until use. Protein concentrations were determined by Bradford assay (Bradford, 1976) using bovine serum albumin as a protein standard.

PPO activity assays were performed as described by Constabel and Ryan (1998) using 3,4-dihydroxyphenylalanine (L-DOPA) as a substrate, with dopachrome production monitored spectrophotometrically at 490 nm (DU 520; Beckman Coulter, Fullerton, CA). The assay buffer contained 100 mM NaPO<sub>4</sub> (pH 7.0), 0.15% SDS, 5 mg·mL<sup>-1</sup> L-DOPA, and 280 U/mL catalase. Parallel assays were performed in the presence of 2 mM Kojic acid (a specific PPO inhibitor) to verify assay specificity (Constabel and Ryan, 1998).

For in-gel PPO activity staining, total protein (10 to 100  $\mu$ g) was separated on a 10% polyacrylamide gel lacking SDS (a native gel). After electrophoresis, the gel was incubated in a staining solution containing 500 mM NaPO<sub>4</sub> (pH 7.0), 3.3 mM L-tyrosine (PPO substrate), and 0.16 mM CuSO<sub>4</sub> for 1 to 16 h for color development.

Browning assays were performed essentially as described by Sullivan et al. (2004). Tobacco or walnut leaves were ground to a fine powder in liquid nitrogen using a mortar and pestle, and the tissue powder was extracted into a buffer containing 20 mM Tris base (pH 7.5) and 100 mM ammonium acetate (3 mL of buffer per gram of tissue). Extracts were centrifuged at 20,000  $g_n$  (4 °C) for 20 min. Supernatants were then incubated at 22 °C for 0.5 to 4 h in the presence or absence of 1 mM L-DOPA, and extract browning was measured spectrophotometrically at 490 nm.

**CLONING AND CHARACTERIZATION OF *jrPPO1*.** Genomic DNA was isolated from ‘Chandler’ walnut leaves as previously described (Dellaporta et al., 1983). To isolate a fragment of a PPO-encoding gene from the DNA, degenerate oligonucleotides were designed based on the highly conserved CuA and CuB copper-binding domains in plant PPOs. The primers Walt 2 (5'-AAR GAR GCI GAY MGI CCI GCI GGI GCI YTI CCI GTN-3') and Walt 45 (5'-GCR TTY TCR TCR TAR AAR AAR AAI SWI SWR TTI ARC CAR TCI GGR TC-3') were used to polymerase chain reaction (PCR) amplify a 0.8-kb DNA fragment from the genomic DNA. This PPO DNA fragment was cloned into the pCR2.1 vector using a TA Cloning Kit (Invitrogen, Carlsbad, CA). The PPO fragment was subsequently excised from the vector by restriction digestion, gel-purified (GeneClean Kit; MP Biomedicals, Solon, OH), and radioactively labeled using a Boehringer Mannheim Random Primed DNA Labeling Kit (Roche Applied Science, Indianapolis, IN). This probe was used to screen a walnut pistillate flower cDNA library as described subsequently.

To generate a cDNA library, total RNA was extracted from field-collected walnut pistillate flowers using the hot borate method (Wan and Wilkins, 1994). PolyA RNA was isolated from the total RNA using an Oligotex mRNA Maxi Kit (Qiagen, Valencia, CA). Starting with 5  $\mu$ g of polyA RNA, a cDNA library was constructed using a Uni-ZAP/Gigapack II Gold cloning kit (Stratagene, La Jolla, CA) according to the manufacturer’s specifications. Plaque hybridization of the cDNA library with the labeled PPO probe (Sambrook et al., 1989) led to the isolation of eight positive phagemids containing cDNA inserts ranging in size from  $\approx$ 1.2 to 2.0 kb. The largest insert was sequenced and included an 1812-bp open reading frame with high homology to plant polyphenol oxidases (see “Results”).

To determine the number of PPO genes in the walnut genome, 8  $\mu\text{g}$  of walnut genomic DNA was separated on a 0.8% agarose gel and subjected to Southern hybridization (Sambrook et al., 1989). A digoxigenin-labeled probe consisting of the full-length *jrPPO1* open reading frame was generated using a PCR DIG Probe Synthesis Kit (Roche Applied Science) and hybridization was performed in DIG Easy Hyb buffer at 42 °C (high stringency) or 37 °C (low stringency) overnight. Washing was performed in 2 $\times$  SSC/0.1% SDS at room temperature and either 0.5 $\times$  SSC/0.1% SDS at 68 °C (high stringency) or 2 $\times$  SSC/0.1% SDS at 58 °C (low stringency). Probe hybridization was visualized colorimetrically using a DIG Nucleic Acid Detection Kit (Roche Applied Science).

**BINARY VECTOR CONSTRUCTION AND PLANT TRANSFORMATION.** Using standard molecular techniques (Sambrook et al., 1989), the *jrPPO1* coding sequence was cloned in the sense orientation into the binary vector pDU92.3103 (Tao et al., 1995). The resulting vector, containing *jrPPO1* driven by a cauliflower mosaic virus 35S promoter, was denoted pDE98.0806. This vector was electroporated into the disarmed *Agrobacterium tumefaciens* strain C58.C1. *Agrobacterium*-mediated leaf disc transformation of tobacco (cv. Petit Havana SR1) was performed as previously described (Horsch et al., 1985). Putative transformants were selected on Murashige and Skoog medium containing 100  $\text{mg}\cdot\text{L}^{-1}$  kanamycin (Murashige and Skoog, 1962), and transformation was confirmed by histochemical analysis of  $\beta$ -glucuronidase activity in leaf tissues (data not shown).

**DISEASE CHALLENGE ASSAYS.** Overnight liquid cultures of *P. syringae* pv. *tabaci* were grown at 30 °C in King's B medium supplemented with 100  $\text{mg}\cdot\text{L}^{-1}$  rifampicin. Cultures were pelleted by centrifugation at 4000  $g_n$  for 10 min and resuspended in 10 mM  $\text{MgCl}_2$  to  $\text{OD}_{600} = 0.5$  [ $2 \times 10^8$  colony-forming units (cfu)/mL]. The suspension was then diluted 1:1000 in 10 mM  $\text{MgCl}_2$  (to  $2 \times 10^5$  cfu/mL) and pressure-infiltrated into the abaxial surface of fully expanded tobacco leaves using a needleless syringe (Katagiri et al., 2002). At various time points postinoculation, in planta bacterial populations were determined by sampling the infiltrated leaf tissue using a cork borer and homogenizing the leaf discs in 10 mM  $\text{MgCl}_2$ . Serial dilutions of the homogenates were plated onto King's B medium with 100  $\text{mg}\cdot\text{L}^{-1}$  rifampicin and then incubated at 30 °C for 48 h for colony counting (Li and Steffens, 2002).

## Results

Previous studies have described PPO activity in walnut hull tissue and heartwood (Cook et al., 1911; Nelson, 1978). To gain a more complete picture of the tissue-level localization of PPO activity in walnut, we isolated protein from non-woody tissues of field-grown walnut and performed spectrophotometric assays of PPO activity using L-DOPA as a phenolic substrate. As summarized in Figure 1A, PPO activity was detected in all green tissues, including leaves, cat-

kins, and pistillate flowers. In walnut fruit, high PPO activity was present in hull tissue, but activity was absent within the shell in pellicle and kernel tissues. As a complement to spectrophotometric PPO activity assays, protein extracts were separated using native polyacrylamide gel electrophoresis and subjected to an in-gel PPO activity stain. A single band of melanin deposition with identical electrophoretic mobility was detected in hull, pistillate flower, catkin, and leaf, suggesting that a single common PPO isoform was expressed in these tissues (Fig. 1B). Because the monophenol tyrosine was used as a substrate for the PPO activity stain, our results also demonstrate that walnut PPO possesses cresolase (monophenol oxidase) activity, unlike many plant PPOs (Steffens et al., 1994).

As discussed previously, in some plant species PPO activity is strongly induced  $\approx 24$  to 72 h after wounding or exposure to methyl jasmonate, a regulatory pattern consistent with a function in plant defense (Constabel and Ryan, 1998). To study the regulation of PPO in walnut, we artificially wounded leaf and hull tissue (on tree) or sprayed the tissues with a 0.025% solution of methyl jasmonate. Forty-eight hours after treatment, total protein was extracted from the tissue and PPO activity assays were performed (Fig. 2). Perhaps unsurprisingly given the high constitutive levels of PPO activity in these tissues, no increase in PPO activity was observed in response to wounding and methyl jasmonate. Considering the reported stability of the PPO enzyme *in vivo* (Dry and Robinson, 1994; Gooding et al., 2001), this result suggests that walnut polyphenol oxidase is not regulated by wounding and methyl jasmonate, at least over the examined 48-h time interval. Instead, a generally small decrease in PPO activity was observed in both leaf and hull tissues.

To pursue further molecular analyses of PPO in walnut, we attempted to clone the PPO-encoding gene(s) from the walnut genome. Using PCR, we amplified from walnut genomic DNA a 0.8-kb fragment that possessed strong sequence similarity to plant PPOs. This DNA fragment was radioactively labeled and used to probe a cDNA library generated from pistillate flower RNA, allowing the isolation of a phagemid with a 2.0-kb cDNA insert. This insert was fully sequenced, and an 1812-bp open reading frame was identified (Fig. 3). The translated open

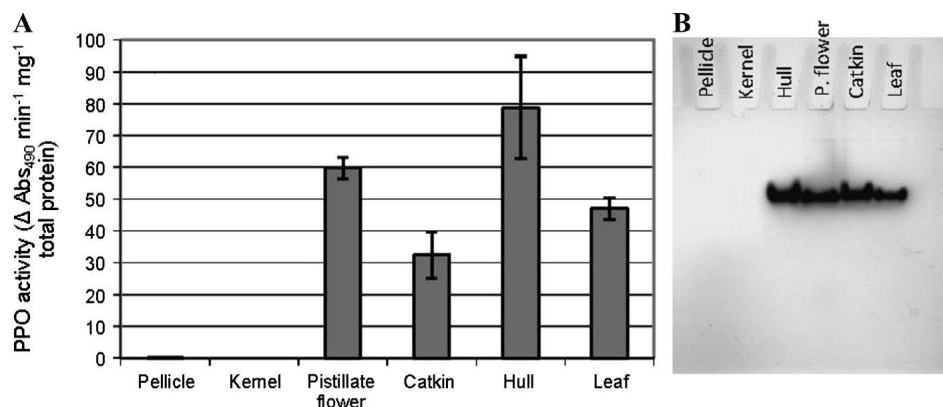


Fig. 1. Tissue localization of polyphenol oxidase (PPO) activity in walnut. (A) PPO activity in walnut tissue extracts was quantified spectrophotometrically, by monitoring the change in absorbance at 490 nm ( $\Delta \text{Abs}_{490}$ ). For each tissue, the mean activity from three biological replicates ( $\pm \text{SE}$ ) is presented. (B) Total protein (10  $\mu\text{g}$ ) from walnut tissue extracts was separated on a native polyacrylamide gel and PPO activity was determined by in-gel tyrosinase staining.

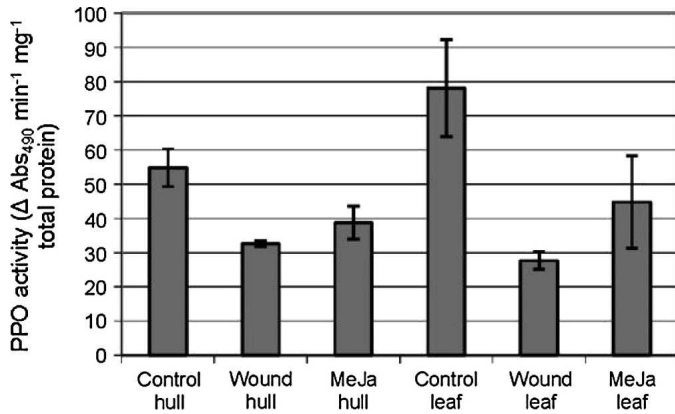


Fig. 2. Regulation of polyphenol oxidase (PPO) in walnut hulls and leaves by wounding and methyl jasmonate (MeJa). Tissues were artificially wounded or sprayed with a 0.025% solution of methyl jasmonate and harvested 48 h after treatment. PPO activity in tissue extracts was quantified spectrophotometrically by monitoring the change in absorbance at 490 nm ( $\Delta \text{Abs}_{490}$ ). For each tissue, the mean PPO activity from three biological replicates ( $\pm \text{SE}$ ) is presented.

reading frame possesses greater than 60% amino acid sequence identity with PPOs from apple, hybrid poplar, grape, and Japanese pear (*Pyrus pyrifolia*) (NCBI accession numbers P43309, AAU12257, CAN61652, and BAB64530, respectively). The N-terminus of the protein shows strong sequence conservation with the N-termini of other plant PPOs and the protein's predicted mass of 67 kDa is typical of plant PPOs, suggesting that the full-length coding sequence has been identified. The gene, denoted *jrPPO1*, possesses CuA and CuB copper-binding domains and is predicted to be chloroplast localized by the subcellular localization prediction programs TargetP and WoLFPSORT (Emanuelsson et al., 1999, 2000; Horton et al., 2007). To determine whether *jrPPO1* is a member of a multigene family in walnut, genomic Southern analysis was performed on isolated walnut DNA. Southern blots performed under both high and low stringency hybridization conditions generated single hybridizing bands, indicating that *jrPPO1* is the only PPO-encoding gene in the walnut genome (Fig. 4). PCR amplification and sequencing of *jrPPO1* from genomic DNA demonstrated that the gene lacks introns (data not shown), which is typical of most plant PPO genes (Thipyapong et al., 2007).

To demonstrate that *jrPPO1* encodes a functional polyphenol oxidase enzyme, we ectopically expressed the gene in the heterologous host tobacco. *jrPPO1* was cloned into the binary vector pDU92.3103 (Tao et al., 1995), generating an expression cassette driven by the strong, constitutive cauliflower mosaic virus 35S promoter. This binary vector was then used to perform *A. tumefaciens*-mediated transformation of tobacco. PPO activity was subsequently quantified in leaf protein extracts from three *jrPPO1*-expressing transgenic tobacco lines. As outlined in Figure 5, several of the transgenic lines displayed five- to 10-fold increases in spectrophotometrically assayed leaf PPO activity compared with wild-type tobacco plants, demonstrating that *jrPPO1* does encode a functional PPO.

If PPOs are important components of plant pathogen defense, then the overexpression of PPO might be expected to increase pathogen resistance. Indeed, Li and Steffens (2002) demonstrated that overexpression of a potato PPO in tomato

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cttatcggttga
L I G -

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Fig. 3. Nucleotide and predicted amino acid sequence of the walnut polyphenol oxidase (PPO) gene *jrPPO1*. The *jrPPO1* open reading frame is 1812 nt and encodes a 67 kDa preprotein possessing greater than 60% amino acid sequence identity to a variety of previously identified plant PPOs. The CuA and CuB motifs are underlined, with copper-binding histidine residues denoted with an asterisk. The putative chloroplast transit peptide cleavage site, as predicted by ChloroP (Emanuelsson et al., 1999), is denoted with an arrowhead.

substantially increased resistance to the bacterial pathogen *P. syringae* pv. *tomato*. Thus, we examined whether expression of *jrPPO1* in tobacco increased resistance to *P. syringae* pv. *tabaci*, a causal agent of angular leaf spot. Leaves from two transgenic tobacco lines displaying the highest PPO activity (10D, 12D) were pressure-infiltrated with a  $2 \times 10^5$  cfu/mL suspension of *P. syringae* pv. *tabaci*, and bacterial growth was monitored by plating diluted leaf extracts at 0 to 4 d

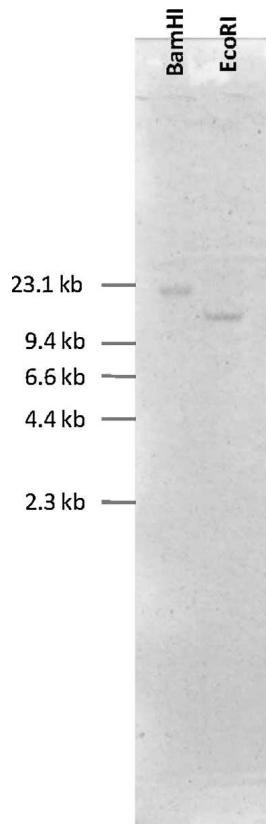


Fig. 4. Southern analysis of walnut genomic DNA to determine polyphenol oxidase (PPO) gene copy number. Eight micrograms of walnut genomic DNA was digested with the BamHI and EcoRI restriction enzymes and size separated on an agarose gel. After capillary transfer to a positively charged nylon membrane, hybridization was performed with a full-length *jrPPO1* probe labeled with digoxigenin. Because neither BamHI nor EcoRI recognition sites are present in the *jrPPO1* sequence, single bands in the Southern analysis indicate a single copy gene. Similar results were obtained using both high and low stringency hybridization conditions (see "Materials and Methods").

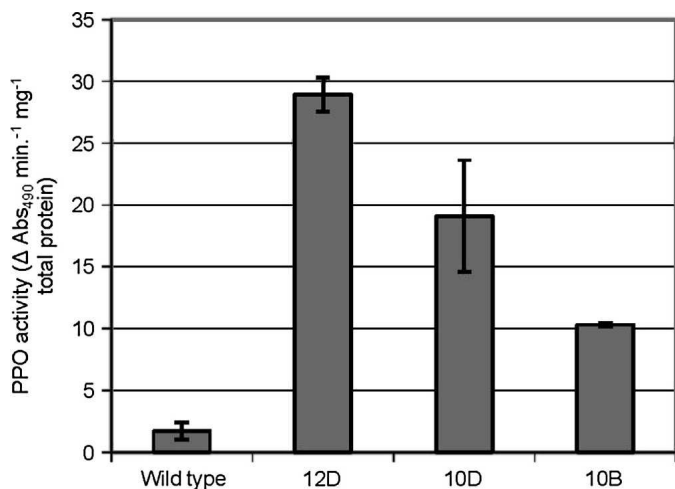


Fig. 5. Polyphenol oxidase (PPO) activity in leaf extracts from transgenic tobacco plants (12D, 10D, 10B) expressing the walnut PPO gene *jrPPO1*. PPO activity was quantified spectrophotometrically by monitoring the change in absorbance at 490 nm ( $\Delta \text{Abs}_{490}$ ). For each line, the mean activity from three biological replicates ( $\pm \text{SE}$ ) is presented.

postinoculation. However, unlike the findings of Li and Steffens (2002), both disease symptoms and bacterial growth in planta were unchanged in the *jrPPO1*-expressing plants (Fig. 6).

Browning assays performed on tobacco leaf extracts showed that extract browning (caused by PPO-mediated quinone formation and polymerization into melanin) occurred only in the presence of exogenously added phenolic substrate (L-DOPA) (Fig. 7). In contrast, walnut leaf extracts display rapid browning in the absence of exogenously added substrates, demonstrating that endogenous phenolic substrates are abundant in walnut leaves (Fig. 7). Thus, any potential antipathogen effects of *jrPPO1* may be limited by the lack of appropriate phenolic substrates in the tobacco experimental system.

## Discussion

Although the use of walnut extracts as hair dyes and skin colorants has a long history (Bittner, 2006), little has been learned about the specific enzymes that underlie these browning processes since Cook et al.'s (1911) initial description of PPO activity in walnut hulls. Our data demonstrate that Cook's "poly-atomic phenol oxidase" is encoded by a single gene, *jrPPO1*, which is constitutively expressed in all green, herbaceous tissues of walnut. Strikingly, PPO-specific activity in walnut tissue extracts is extremely high compared with characterized PPO activity in other plant species. Basal PPO

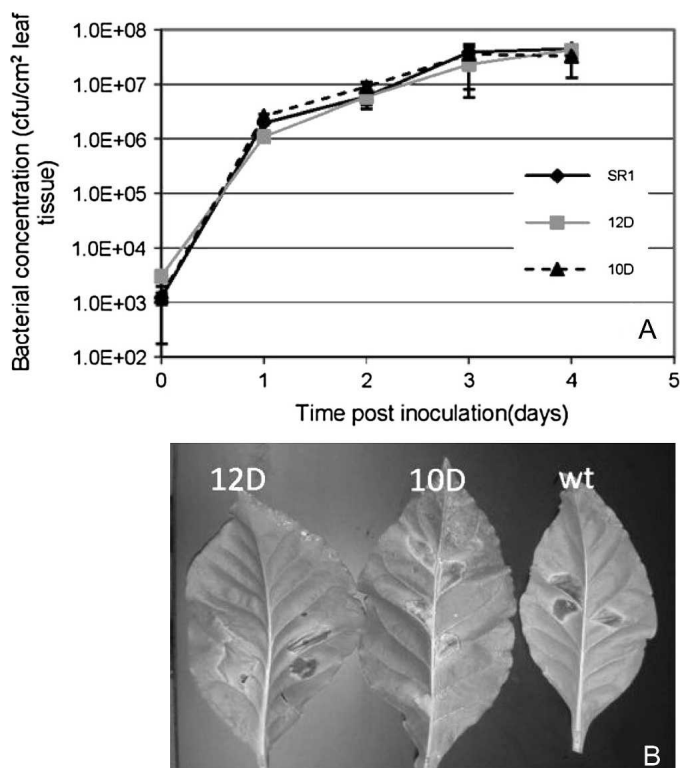


Fig. 6. Expression of the walnut polyphenol oxidase (PPO) gene *jrPPO1* in transgenic tobacco plants does not confer increased resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tabaci*. Fully expanded leaves of wild-type (wt) and *jrPPO1*-expressing transgenic tobacco lines (10D, 12D) were infiltrated with a  $2 \times 10^5$  cfu/mL suspension of *P. syringae* pv. *tabaci*. (A) In planta bacterial populations were quantified by plating diluted extracts from inoculated tissue. Data points represent mean bacterial concentrations  $\pm \text{SE}$  for three biological replicates. (B) Disease symptom development in the leaves of wild-type, 10D, and 12D tobacco plants is shown.

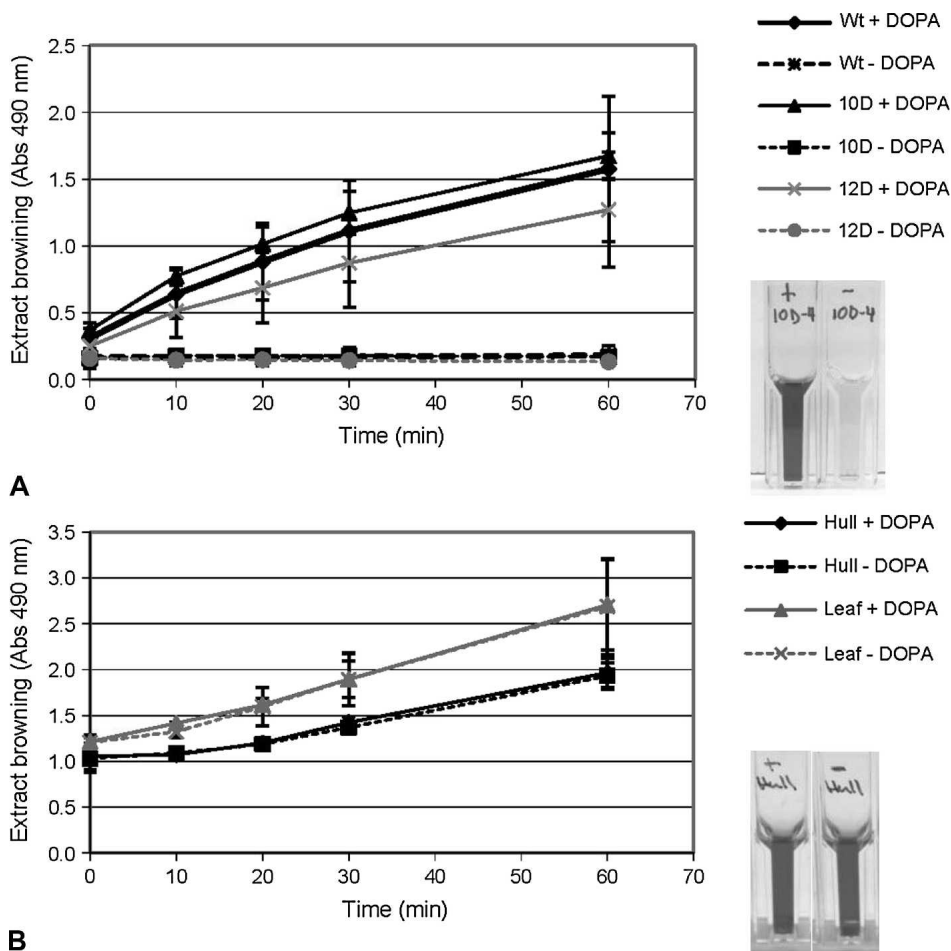


Fig. 7. Tobacco leaves lack endogenous substrates for the walnut polyphenol oxidase *jrPPO1*. Freshly isolated protein extracts from 1 g of tobacco leaves (A) or walnut hulls/leaves (B) were incubated in the presence or absence of 3,4-dihydroxyphenylalanine (L-DOPA) for the time periods indicated. Extract browning was monitored by measuring absorbance at 490 nm. Data points represent mean absorbance  $\pm$  SD from two biological replicates. Insets demonstrate typical results after a 60-min incubation with + and - indicating the presence and absence of L-DOPA, respectively.

activity in walnut leaves was more than 10 times higher than the PPO activity reported in the leaves of wounded (induced) tobacco and hybrid poplar plants and is comparable to the highest PPO activity observed in a survey of 18 crop plants (leaves of tomato plants treated with methyl jasmonate) (Constabel and Ryan, 1998). To our knowledge, high constitutive expression of PPO has been described only in soybean (*Glycine max*) and willow (*Salix* spp.) and is in contrast to the moderate or dramatic wound induction of PPO in most plant species (Constabel and Ryan, 1998). Although significant evidence supports a bacteriocidal and/or insecticidal role for PPO in species such as hybrid poplar and tomato, constitutive expression is not generally associated with disease and pest resistance genes as a result of large associated fitness costs (Heidel et al., 2004). Thus, the native physiological role of highly expressed PPOs in species such as walnut, willow, and soybean remains unclear.

As described, the expression of *jrPPO1* in transgenic tobacco plants increased leaf PPO activity greater than 10-fold (line 12D) but had no effect on susceptibility to the pathogen *P. syringae* pv. *tabaci* (Figs. 5 and 6). However, it is not PPO protein per se, but the reactive quinones generated by

PPO activity, that are thought to play a role in inhibiting pathogen/insect growth in some plants (Steffens et al., 1994). Thus, both PPO and abundant phenolic substrates are presumably required for PPO to fulfill a role in defense, and these endogenous phenolic substrates are clearly lacking in tobacco leaves under the tested conditions. This lack of appropriate phenolic substrates in tobacco leaves likely explains the contrast between our results and the work of Li and Steffens (2002), who found that overexpression of PPO in tomato increased bacterial disease resistance. A recent examination of quinone-mediated inhibition of proteolysis during ensiling of alfalfa parallels the results of our studies with *jrPPO1*-expressing tobacco. In this work, expression of a red clover (*Trifolium pratense*) PPO in alfalfa (*Medicago sativa*) did not inhibit postharvest proteolysis unless phenolic substrates were exogenously supplied, reflecting a lack of native PPO substrates in alfalfa plants (Sullivan et al., 2004). Thus, transgenic approaches to the use of PPO in plant improvement are probably best applied between closely related species [as in Li and Steffens' (2002) work with potato and tomato], in which appropriate phenolic substrates are more likely to be present in the plant host.

To continue our examination of the potential physiological functions of PPO in walnut (where *jrPPO1* substrates are abundant, see Fig. 7), we recently generated transgenic walnut plants expressing a self-complementary *jrPPO1* transgene designed to silence PPO in planta. Future studies characterizing the basic physiology, growth, and susceptibility to disease and insect pests of these PPO-silenced walnuts should provide valuable further information about the functional role of the highly abundant PPO enzyme in walnut.

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